

9/PR13

09889993.100501
09/889993
JC18 Rec'd PCT/PTO 23 JUL 2001

M 4452

Mammal, Method for Producing Same and Its Use

Ins 61)

The present invention relates to a mammal in which a ductal carcinoma *in situ* (DCIS) of the female mammary gland can be induced. The invention also relates to a method of producing such a mammal and its use for studying DCIS and/or its progression towards an invasive ductal carcinoma of the breast, and for the development of diagnostic and/or therapeutic means for this purpose.

The mammary carcinoma which occurs with a frequency of about 10 % among the female population is one of the pressing health problems of our time. About 80 % of the mammary carcinomas are invasive ductal mammary carcinomas. Mammary tumors belonging to the type of ductal carcinoma *in situ* (DCIS) are frequently diagnosed in a mammography. DCIS is characterized by a non-invasive neoplastic proliferation, i.e. a proliferation not yet breaking through the basal membrane, of epithelial cells into the lumen of the ductulo-lobular unit of the mammary system. A DCIS may develop into an invasive ductal mammary carcinoma. The molecular causes of this are, however, not known. Likewise, a prediction as to whether and when such a development occurs is not possible either. As a result, radical mastectomy, i.e. complete removal of the female breast and local lymph nodes, is usually carried out when DCIS of the female breast is diagnosed. Estimates, however, show that about 60 % of the radical mastectomies represent an excessive treatment.

It is thus the object of the present invention to provide a product by which the molecular causes of DCIS, in particular its progression towards an invasive ductal mammary carcinoma, can be studied and optionally possibilities for a reliable diagnosis and/or appropriate therapy can be shown.

According to the invention this is achieved by the subject

matters defined in the claims.

The present invention is based on Applicant's insights that DCIS of the female mammary gland can be induced in mammals, e.g. mice, containing an oncogene, e.g. the early region of SV40, i.e. the gene for the SV40 T-Ag, which can be activated by lactotropic hormones such as estrogen, prolactin, insulin and hydrocortisone. He also found that DCIS may develop into an invasive ductal mammary carcinoma.

According to the invention, Applicant's insights are used to provide a mammal having an inducible DCIS of the female mammary gland, which contains an oncogene that can be activated by lactotropic hormones. DCIS may preferably develop into an invasive ductal mammary carcinoma.

The expression "DCIS of the female mammary gland" refers to a non-invasive neoplastic proliferation, i.e. a proliferation which does not yet break through the basal membrane, of epithelial cells into the lumen of the ductulo-lobulary unit of the mammary gland system. In particular, DCIS distinguishes itself by histological features, such as hyperchromatic, pleomorphous, large-scale structured or strikingly large nuclei. It may show a shifted nucleus-plasma relation or numerous mitotic patterns. DCIS may also be characterized in that the proliferation of the epithelial cells into the lumen of the duct manifests itself as a multi-layered or sievelike lining or as an intraluminal branching or by means of micropapillae. Moreover, DCIS may show as necroses, apoptosis patterns, psammoma bodies, i.e. onion skin-like crystallization products having calcifications, in the lumen of the duct and loss of the myoepithelial layer underneath the basal membrane.

The expression "lactotropic hormones" refers to hormones which are released by mammals, e.g. during pregnancy and/or lactation, and have a lactotropic effect. Examples of such hormones are estrogen, prolactin, insulin and hydrocortisone.

The expression "oncogene" comprises any gene or portions thereof which may have a cell-transforming property. Examples of such genes are erb A, erb B, fos, myc, E6, E7 and the early region of SV40, i.e. the gene for SV40 T-Ag, as well as mutated p53. The oncogene may also comprise sequences coding for a strong, i.e. immunodominant, T-cell epitope, e.g. the MHCI-restricted epitope n118 of the LCM virus nucleoprotein.

SUBC1) The expression "oncogene that can be activated by lactotropic hormones" refers to the fact that the above oncogene can be activated by lactotropic hormones. This may be achieved in the most differing ways. It may be favorable for the oncogene to be controlled by a promoter which is specific to one or more lactotropic hormones. Such a promoter is e.g. the "whey acidic protein" (WAP) promoter. Its specificity comprises the lactotropic hormones estrogen, prolactin, insulin and hydrocortisone. Reference is made to the below description regarding the production of a mammal according to the invention.

The expression "mammal" comprises any animals, with the exception of humans, which release lactotropic hormones, e.g. during pregnancy and/or lactation, and which may contain an oncogene that can be activated by lactotropic hormones. Examples of such mammals are mice, rats, rabbits, horses, bovine animals, sheep, goats, monkeys, pigs, dogs and cats, mice being mentioned above all.

SUBC2) Preferred mammals of the present invention are mice which contain the gene for the SV40 T-Ag under the control of the WAP promoter. The SV-40 T-Ag gene may also contain sequences coding for a strong, i.e. immunodominant, T-cell epitope, e.g. epitope n118 of the LCM virus nucleoprotein. Such mice are referred to as WAP-T or WAP-T-NP (cf. figure 1). The mice WAP-T-1, WAP-T-2, WAP-T-10, WAP-T-NP6, WAP-T-NP8 and WAP-T-NP10 are preferred. These mice are distinguished as follows:

WAP-T-1

These mice usually develop multifocal invasive ductal mammary

carcinoms after an average of 7 months following the induction with lactotropic hormones. No preferential tumor formation can be observed in one of the mammae. Invasive carcinomas are, as a rule, differentiated tubularly to papillarily and have partially solid anaplastic portions which may also show a desmoplastic reaction. Pulmonary metastases occur occasionally. However, the majority of animals does not form metastases, and the primary tumors only grow slowly. Macroscopically non-affected mammae show multifocal DCIS. Due to the isomorphous manifestation of the nuclei most DCIS may be classified as "low grade" in analogy to the classification of human DCIS according to the NUYS index. Comedonecroses and psammoma bodies are observed from time to time. However, there is a formation of intraluminal branches ("roman arches" formation) (cf. figure 4).

WAP-T-2

These animals have a light-brown coat. Furthermore, they develop invasive ductal mammary carcinomas about 6 months after the induction with lactotropic hormones. Differentiated mammary carcinomas are predominantly tubular to lobular. Anaplastic mammary carcinomas appear with some tumor giant cells. Micrometastases in lymph nodes are observed. In rare cases, fibrosarcomas may form, starting from mammary or uterus. The multifocally occurring DCIS show micropapillary and cribriform growth patterns. Forms which have monolayered lining and comedonecrosis or psammoma bodies are also observed. Since the cell nuclei are usually isomorphous, these DCIS cannot be assessed as "high grade" (cf. above) (cf. figure 5).

WAP-T-10

About 8 months after the induction with lactotropic hormones, these mice develop palpable ductal mammary carcinomas which may form metastases. Both solid carcinomas and carcinomas with little differentiation which have numerous mitoses as well as tubular to papillary forms may be found. The investigated metastases are differentiated papillarily. The multifocally occurring DCIS have comedonecroses and due to

the morphology of nuclei (pleomorphism, hyperchromasia, and others) correspond to a "high grade" DCIS (see above; and figure 6).

WAP-T-NP6

These mice develop palpable invasive carcinomas about 11 months after multiple induction with lactotropic hormones. In rare cases, hepatocellular adenomas and adenomas of the salivary gland occur as well. The mammary carcinomas are differentiated predominantly tubularly or papillarily, in some cases they are differentiated only moderately with extensive necroses and they are partially also solid. Micrometastases occur in mammary lymph nodes. The cell nuclei of the multifocally occurring DCIS are usually inconspicuously "low grade" (see above) and comedonecroses are present (cf. figure 7).

WAP-T-NP8

These mice develop invasive ductal carcinomas about 5 months following induction with lactotropic hormones. Both tubulo-papillarily differentiated and poorly differentiated solid tumors are found. Animals having poorly differentiated tumors show pulmonary metastases. The lumens of localized small invasive carcinomas and of DCIS are in some cases infiltrated by granulocytes. Non-epithelial tumors of mammary origin, e.g. fibrosarcomas and osteosarcomas, and infiltrating histiocytic sarcomas also occur occasionally. The multifocal DCIS apparent 15 to 20 weeks following induction with lactotropic hormones correspond to a "high grade" form due to the morphology of cell nuclei (see above; and figure 8).

WAP-T-NP10

These mice develop an invasive ductal mammary carcinoma about 11 months following several inductions with lactotropic hormones. These carcinomas are frequently differentiated tubularly to papillarily and have solid and necrotic but only moderately differentiated portions. The DCIS appear with isomorphous nuclei (not "high grade"; see above). DCIS having micropapillary growth and forms with total loss of the

myoepithelial layer and psammoma body formation occur (cf. figure 9).

Another subject matter of the present invention relates to cells which are obtained from the mammal according to the invention. These cells may be present in any form, e.g. in a primary or long-term culture.

A mammal according to the invention may be provided by common methods. A method may be favorable which comprises the steps of:

- (a) introducing a DNA coding for an oncogene into inseminated oocytes of a mammal, the DNA being controlled by a promoter specific to lactotropic hormones,
- (b) implanting the oocytes from (a) into pseudo-pregnant mammals, and
- (c) selecting the progeny obtained in (b) for the formation of a DCIS.

As to the expressions "oncogene", "lactotropic hormones", "mammals" and "DCIS" reference is made to the above explanations.

Furthermore, the expression "pseudopregnant mammals" refers to mammals which were paired with non-potent, i.e. sterile or vasectomized, male mammals, and have a vaginal plug. Reference is made to the below example.

The expression "inseminated oocytes" refers to oocytes of pregnant mammals which have been emptied. Reference is made to the below example.

The expression "DNA coding for an oncogene and controlled by a promoter specific to lactotropic hormones" relates to a DNA present in any form and having these properties. The DNA may be present as such or in combination with another DNA, e.g. a vector. It may also be circular or linear. Furthermore, it

Figure 1 shows a DNA used for the production of a mouse

according to the invention and coding for the SV40 T-Ag or the SV40 T-Ag containing the n118 epitope of the LCM virus (SV40 T-Ag-NP). The DNA is used in a linear form and injected into inseminated oocytes.

Figure 2 shows the expression of SV40 T-Ag or SV40 T-Ag-NP in the mammary glands of mice according to the invention.

- (a) Nuclear expression of SV40 T-Ag in the epithelial cells of morphologically inconspicuous ductuli 12 months following the induction of the WAP-T-1 mouse according to the invention.
- (b) Nuclear expression of T-Ag in the tumor cells of DCIS 12 months following the induction of the WAP-T-NP6 mouse according to the invention.

Figure 3 shows examples of frequent tumor phenotypes of mice according to the invention. Sections embedded in paraffin, H&E staining;

- (a) Invasive ductal mammary carcinoma differentiated tubularly to papillary and with desmoplastic reaction of the WAP-T-10 mouse.
- (b) Anaplastic invasive ductal mammary carcinoma with extensive necroses of the WAP-T-NP8 mouse.

Figure 4 shows induced DCIS with relatively inconspicuous nuclei in the DCIS mouse WAP-T-1, in part with intraluminal branching, psammoma bodies and comedonecrosis.

Figure 5 shows in the DCIS mouse WAP-T-2 induced DCIS with micropapillary growth pattern, desmoplastic reaction and inflammatory infiltrates, in addition a DCIS with monolayered to multi-layered lining as

well as psammoma bodies and comedonecrosis.

Figure 6 shows several induced DCIS with hyperchromatic pleomorphous nuclei in the DCIS mouse WAP-T-10. Inflammatory infiltrates can be detected in the vicinity.

Figure 7 shows an induced DCIS with usually inconspicuous nuclei and occasional comedonecroses in the DCIS mouse WAP-T-NP6. Mono-layered and locally multilayered linings of the ductal lumens are detectable.

Figure 8 shows several induced DCIS with pleomorphous, partially strikingly large nuclei and psammoma bodies in the DCIS mouse WAP-T-NP8.

Figure 9 shows an induced DCIS with a growth pattern forming relatively isomorphous nuclei and micropapillary and partially intraluminal branchings in the DCIS mouse WAP-T-NP10.

The invention is explained by the example.

Example: Production of a mammal according to the invention

About 20 female CB6F1 mice at the age of 4 to 5 weeks are superovulated by intraperitoneal injection of 5 U PMS (pregnant mare's serum) on day 1 and another intraperitoneal injection of 5 U hCG (human chorionic gonadotropin) on day 3 and are paired with male CB6F1 animals in the evening of that very day. In the morning of the 4th day, the animals are investigated for the presence of a vaginal plug, positive animals are killed by cervical dislocation and the oviducts are removed. The oocytes are removed from the oviducts and placed into M2 medium, the cumulus cells are separated by short incubation using hyaluronidase, the oocytes are washed thoroughly and stored in an incubator (5 % CO₂, 85 % humidity, 37°C) in M16 medium covered with paraffin oil until they are

microinjected.

The DNA of figure 1 is usually injected into the male pronucleus of inseminated oocytes on the 4th day. Injected oocytes are then incubated in the incubator up to the retransfer taking place the following day. For providing pseudopregnant foster mice, about 25 female B6CBAF1 mice at the age of 8 to 12 weeks are paired with vasectomized male mice in the evening before the microinjection. In the morning of the 5th day, the animals with vaginal plug are selected for retransfer of the injected oocytes. The microinjected oocytes cultured overnight and proliferated to the two-cell stage are reimplanted on the 5th day. In this connection, 10-15 embryos are rinsed into the infundibulum of an oviduct of a narcotized foster mouse. 19-20 days after the retransfer the implanted embryos are born. The mice shown in figures 4-9 are obtained.